Introduction to Multiplex and Nested PCR

Multiplex PCR is a PCR reaction where two or more targets are detected in the same reaction. This method is simple and can be less expensive than conducting multiple single product reactions since one reaction mix is used to amplify multiple targets. Very often the multiplex assay includes an internal (positive) control – a PCR target added to the reaction or derived from the sample used. Amplification of the internal control along with the target of interest indicates that PCR reaction works as expected even in the absence of a second, more specific PCR procedure. In plant pathogen diagnostics multiplex PCR internal control primers often target nucleic acid (NA) from the host plant. Thus amplification of the internal control confirms the presence of amplifiable NA and validates the NA extraction procedures. More importantly amplification of the internal control along with lack of amplification of the pathogen specific product confirms that negative PCR result for the pathogen is due to the absence of target detectable not to poor NA extraction or reaction inhibition.

Nested PCR is a PCR that consists of 2 or more PCR reactions conducted in series where the PCR product of the first reaction (round) serves as template for the second reaction (round) of PCR and so on. For example, the DNA target in the second round nested reaction is found within the target amplified in the first reaction, and thus the product of the nested PCR is always smaller than the product of the first step. Nested PCR is used for two main reasons: first to increase specificity and second to increase sensitivity

The Nested PCR assay used for *P. ramorum*

A nested PCR procedure was developed by Hayden *et al.* (2004) to amplify *P. ramorum* specifically. APHIS-PPQ-CPHST modified the procedure slightly and validated it as the approved conventional PCR assay for *P. ramorum* diagnostics. This assay has been in use within PPQ-CPHST since 2003 (Levy and Mavrodieva, 2003) and has been the primary method for detecting and diagnosing samples that contain *P. ramorum.* It is highly sensitive (can detect >10fg of target DNA under test conditions). However, since 2003, additional *Phytophthora* species have been identified that can cross react with the primers used in this assay. (Please read the caution statement on page 10 for some caveats in interpreting presumptive positive results of this nested PCR assay.)

The Multiplex PCR assay used for P. ramorum

The Multiplex PCR procedure used in our diagnostic procedures for detection of *P. ramorum* was originally developed as a diagnostic PCR assay for *P. lateralis*. However, this assay also amplifies a PCR product for the closely related *P. ramorum* and *P. hibernalis* (Winton & Hansen, 2001), and for the recently identified *P. foliorum* (species description in press). The multiplex procedure is a minor variation on the published procedure of Winton and Hansen. It is *not* used as a primary diagnostic for *P. ramorum* for two reasons. First, it has a lower sensitivity for detecting *P. ramorum* than the Nested PCR. Second, it is known to cross-react to produce identically-sized PCR products with DNAs from all four closely related species previously mentioned. Its usefulness stems from the fact that the internal (positive) control product (primed by NS1/NS2) amplifies both plant and fungal DNAs, and it can directly assess whether the DNA is of sufficient quality /quantity to permit a robust PCR assay. Amplification of the internal control product along with lack of amplification of the *Phytophthora* product confirms that the negative result for the pathogen is due to the agent (target) absence not failed PCR reaction or inhibitors.

1. Materials and Equipment

a. Equipment needed:

- Conventional PCR instrument (Biometra® or other)
- Dedicated, annually-calibrated pipettors (P10, P50, P200)
- Sterile filter (barrier) pipette tips (P10, P50, P200)
- 1.5ml micro centrifuge tubes (for making dilutions and master mixes, any vendor)
- Thin-wall 0.2ml PCR tubes (any vendor)

b. Reagents needed:

- dNTP Mix (10 mM solution, PCR reagent, D-7295, Sigma)
- Molecular Grade Water (any vendor)
- 100 base pair DNA ladder (BioVenture M1 or other vendor)
- Platinum *Taq* DNA polymerase (10966-034, Invitrogen,

Note: Platinum *Taq* is supplied in a set with 10X PCR Buffer and 50mM $MgCl_2$

2. Primers needed

Multiplex Primers:

Primer Name	**Primer Sequence** (synthesized by Integrated DNA Technologies, Inc., Purification - Standard Desalting)
Platf primer	5'-TTAGTTGGGGGCTTCTGTTC-3'
Platr primer	5'-AGCTGCCAACACAAATTTC-3'
NS1 primer	5'-GTAGTCATATGCTTGTCTC-3'
NS2 primer	5'-GGCTGCTGGCACCAGACTTGC-3'

Nested Primers:

Primer Name	**Primer Sequence** (synthesized by Integrated DNA Technologies, Inc., Purification - Standard Desalting)
Phyto1 primer	5'-CATGGCGAGCGCTTGA-3'
Phyto2 primer	5'-AAAGCCAAGCCCTGCAC-3'
Phyto3 primer	5'-GGTGGATGGGGACGTG-3'
Phyto4 primer	5'-GAAGCCGCCAACACAAG-3'

3. Reagent Preparation

a. Primer solutions

Prepare working solutions of each of the PCR primer pair mixtures as indicated below. Tubes with lyophilized primers are centrifuged briefly (10-20 seconds at 10,000 rpm) before opening to ensure that the lyophilized material is in the bottom of the tube. Primers are re-hydrated to 100µM concentration in molecular-grade H_2O and stored at - 20°C

Primer Name	Re-hydrated Conc.	Stock Conc.	Working Conc.
Platf	100 µM	40 µM	4 µM
Platr	100 µM	40 µM	4 µM
NS1	100 µM	10 µM	1 uM
NS2	100 µM	10 µM	1 uM
Phyto 1	100 µM	50 µM	5 µM
Phyto 2	100 µM	50 µM	5 µM
Phyto 3	100 µM	50 µM	5 µM
Phyto 4	100 µM	50 µM	5 µM

- 50µM stocks (100×) of a primer mix of Phyto1 and Phyto4, and of Phyto2 and Phtyo3 are made from these 100µM freezer stocks and stored at - 20°C.
- 10µM stocks (100×) of a primer mix of NS1and NS2, and 40µM stocks (100×) of a primer mix of Platf and Platr are made from these 100µM freezer stocks and stored at - 20°C.
- 5µM working mixes of primers Phyto1 / Phyto4, and of Phyto2 / Phtyo3 are made (10× final concentration) from the 100× stocks and stored at - 20°C
- 4µM working mixes of primers PlatF / PlatR are made (10× final concentration) from the 100× stocks and stored at - 20°C.
- 1µM working mixes of primers NS1 / NS2 are made (10× final concentration) from the 100× stocks and stored at - 20°C.

4. Sample Preparation

- Determine the number of samples to be tested. On a clean (decontaminated) work surface, label a set of 1.5ml microcentrifuge tubes that will be used for a 1:100 sample dilution in molecular grade water.
- Label 2 sets of 0.2ml PCR reaction tubes, one for Nested and one for Multiplex PCR reactions, and include 2 tubes each: one for the non-template control that contains molecular grade dH_2O instead of DNA and another for the *P. ramorum* positive control.
- Thaw DNA samples to be tested (if frozen). Vortex the sample DNAs for 2-3 seconds at a speed setting of 7 (scale 0 – 10), and spin them briefly (10-20 seconds at 10,000 rpm) in a bench top microcentrifuge to prevent aerosols. Place the sample tubes on ice.
- Make a 1:100 dilution using 2µl of sample (environmental) DNA. Dilute the DNA in molecular grade water (2µl sample: 198µl H_2O). Use this diluted sample to run both the Multiplex and 1st-round Nested reactions

5. Master Mix Preparation

- Thaw the 10x PCR buffer, 50mM $MgCl_2$, primer stock and dNTPs. Vortex for 5-10 seconds at a speed setting of 7, and spin them briefly (10-20 seconds at 10,000 rpm) in a bench top micro centrifuge to settle the liquid to the bottom of the tube. Then place in ice. Note: *Taq* Polymerase should be kept on ice after it's taken out of the freezer.

- In a decontaminated PCR clean station (or bio-safety cabinet) prepare 2 master mixes, one for each PCR assay type.
- Calculate the quantity of the master mix to include the number of the samples to be assayed, plus 2 extra reactions for the positive (DNA) controls and for the 1 negative (H_2O, non-template) controls, plus approximately 10% extra reaction mix (i.e.: 1-2 samples for 10-20 reactions) to ensure sufficient volume for all samples.
- Keep the master mixes on ice until dispensed into the pre-labeled 0.2ml reaction tubes.
- Ensure that each master mix is thoroughly mixed by slowly pipetting up and down several times.

Multiplex Reaction Mixture (25 microliter reaction size)

Reagent	Volume (1 reaction)
10×PCR Reaction Buffer (provided with *Taq* polymerase)	2.5µl / rxn
10 mM mixture dNTPs	0.5µl / rxn
50 mM $MgCl_2$ (provided with *Taq* polymerase)	0.75µl / rxn
4 µM mixture primers Platf / Platr	2.5µl / rxn
1 µM mixture primers NS1 / NS2	2.5µl / rxn
5u/µl Platinum *Taq* Polymerase (Invitrogen)	0.25µl / rxn
H_2O (molecular grade)	10µl / rxn

Aliquot the master mix (19.0µl/tube) into corresponding 0.2ml PCR tube.

Nested Reaction Mixture, 1st Round (25 microliter reaction size).

Reagent	Volume (1 reaction)
10×PCR Reaction Buffer (provided with *Taq* polymerase)	2.5µl / rxn
10 mM mixture dNTPs	0.5µl / rxn
50 mM $MgCl_2$ (provided with *Taq* polymerase)	1.0µl / rxn
5 µM mixture primers Phyto1/Phyto4	2.5µl / rxn
5u/µl Platinum *Taq* Polymerase (Invitrogen)	0.25µl / rxn
H_2O (molecular grade)	12.25µl / rxn

Aliquot the master mix (19.0µl/tube) into corresponding 0.2ml PCR tube.

Nested Reaction Mixture, 2nd round (25 micro liter reaction size).

Reagent	Volume (1 reaction)
10×PCR Reaction Buffer (provided with *Taq* polymerase)	2.5µl / rxn
10 mM mixture dNTPs	0.5µl / rxn
50 mM $MgCl_2$ (provided with *Taq* polymerase)	1.0µl / rxn
5 µM mixture primers Phyto2/Phyto3	2.5µl / rxn
5u/µl Platinum *Taq* Polymerase (Invitrogen)	0.25µl / rxn
H_2O (molecular grade)	12.25µl / rxn

Aliquot the master mix (19.0µl/tube) into corresponding 0.2ml PCR tube.

Remove the rack of the reaction tubes containing the master mixes from the PCR clean-station or bio-safety cabinet and move into the room containing the PCR machine(s).

6. Addition of Sample or Control to Mix

a. **Multiplex and 1st-round Nested reactions**:

Working on a new disposable mat, add 6.0µl of each 1:100 sample DNA dilution into each of the designated tubes (total volume 25µl). Then add 6.0µl molecular grade H_2O for the negative (H_2O, non-template) control and 6.0µl of the positive control DNA for each positive control reaction. Change gloves between handling the sets of positive control DNAs for the two assays.

b. **2nd-round Nested reaction**:

Make a 1:500 dilution of the first-round PCR reaction (1 µl 1st round PCR reaction plus 499 µl molecular grade water). Vortex each dilution for 5-15 sec at speed setting 7 and spin tubes briefly (10-20 sec at 10,000 rpm) in a bench top micro centrifuge. Store this dilution on ice if not being added immediately into 2nd round reactions. Add 6.0µl of each 1:500 dilution into the corresponding tube (total volume 25µl). Add 6.0µl molecular grade H_2O to the negative (H_2O, non-template) control and 6.0µl of the second-round positive control DNA to the positive control reaction.

7. Thermocycler information

- Turn on the thermocycler and allow the machine to run through its self-testing procedures.

- For more information on instrument set-up see the user's manual for the thermocycler to be used.
- Place the PCR tubes into the selected PCR machine and start the run.

Thermocycler settings for Multiplex PCR

- **Settings:**

Step 1) 94 C for 1 minute, 25 seconds
Step 2) 93 C for 35 seconds
Step 3) 52 C for 30 seconds
Step 4) 72 C for 1 minute
Step 5) Go to step 2, repeat 34 times
Step 6) 72 C for 10 minutes
Step 7) 4 C hold

Note: Use maximum ramping temperatures between steps

Thermocycler settings for Nested PCR

- **Use these settings for both 1st and 2nd rounds of Nested PCR**
- **Settings:**

Step1) 94 C for 1 minute, 25 seconds
Step 2) 92 C for 35 seconds
Step 3) 62 C for 55 seconds
Step 4) 72 C for 50 seconds
Step 5) Go to step 2, repeat 34 times
Step 6) 72 C for 10 minutes
Step 7) 4 C hold

Note: Use maximum ramping temperatures between steps

8. Gel Electrophoresis

- Prepare a 1.5% agarose (Ultra Pure™ Agarose, Invitrogen, Cat #15510-027) gel(s) in 1x TAE buffer. Gels should be sufficient size to accommodate the number of samples, plus controls. Include two lanes with DNA ladder flanking the PCR samples.

- Load the Multiplex and Nested PCR products for the same samples onto the same gel by loading the Nested reaction in the upper gel lanes and

the Multiplex reaction in the bottom lanes. This loading design facilitates the direct comparison of the results from these two assays.

- Mix 12µl of the 25µl total reaction with 3µl, 6×loading buffer. Mix by pipetting up and down and load mixture into the gel.
- Prepare DNA ladder samples as follow: Mix 3µl of 100bp DNA ladder (BioVentures) with 9µl of dH2O and 3µl of 6×loading buffer per sample. Load on both sides of the PCR samples.
- Negative (H_2O, non-template) and positive controls should be loaded after the DNA ladder on the right.
- Gels are run in 1x TAE buffer for 1 hour at 100V (constant).
- Stain the gels for 10-15 minutes in EtBr (5 mg/ml) solution and destain 10-15 minutes in ddH_2O, using a horizontal orbital shaker.
- Document the assay results using a digital imaging system.
- Dispose of the gel, any contaminated gloves and paper toweling, into the EtBr hazardous waste receptacle. Note: EtBr is a hazardous chemical. Please read the MSDS sheet before using it.
- Shut down the digital imaging system if you are the last individual to be using it that day.

9. Assessment of Samples

Nested PCR assessment

1. Is each ladder distinct and well resolved?
   If no, rerun the gel with the remainder of the reaction.

2. Does the nested PCR negative control lane contain any band(s)?
   If yes, the PCR is invalid and the nested PCR must be repeated.

3. Does the nested PCR positive control have a band of predicted size (291 bp)?
   If no, the PCR is invalid and the nested PCR must be repeated.

Note: An environmental sample or genomic DNA used as a *P. ramorum* positive control should be evaluated BEFORE using it as an assay positive control and must produce a well-defined, 291-bp band.

4. Did the sample produce a band of 291 bp in the Nested reaction?

a) Yes: If a 291-bp band is present in the nested reaction, the sample must be retested in second nested PCR at both 1:10 and 1:100 dilutions to verify the first run results.

b) No: If there is no band in the nested reaction, the sample is considered to test negative for *P. ramorum*. *However, see page 10 for a possible caveat to this result.*

Multiplex PCR assessment

1. Do the ladders look normal?
   If no, rerun the gel with the remainder of the reactions.

2. Does the multiplex PCR negative control lane contain any bands?
   If yes, the test is invalid and the multiplex PCR must be repeated.

3. Does the sample have an internal control band of predicted size (~550 bp) for the Multiplex reaction?
   a) Yes: This indicates the DNA is acceptable quality to permit an accurate determination.
   b) No: If there is no internal control band for the sample, the sample must be retested at 1:10 and 1:100 dilutions. If after retesting from both dilutions there is still no well-resolved internal control band, then the sample fails quality control and DNA must be re-isolated in order to complete a diagnosis.

4. Does the sample have a *Phytophthora* sp. specific band (~738bp) for the Multiplex reaction?
   If Yes: This will indicate that the sample is positive for *Phytophthora spp*. that includes *P. foliorum P. hibernalis*, *P. lateralis* and *P. ramorum*.

   Note: If a sample produces a *Phytophthora* sp. specific band in the Multiplex PCR and no Nested band of ~291bp, it should be retested at both 1:10, and 1:100 dilutions in both the Nested and Multiplex PCR assays prior to completing a diagnosis.

Positive sample determination

A sample must be positive (291 bp) in the nested PCR **and** have an internal control band (~550 bp) in the multiplex PCR to be considered positive. It does not necessarily need to be positive for the specific band (~738bp) in the multiplex to ultimately be determined to be positive since the multiplex assay is known to be less sensitive.

When a sample has tested positive in the nested PCR two times, it may be considered to be positive for *P. ramorum*. Alternatively, the sample can be retested by the Real-Time PCR assay for *P. ramorum*, or the Nested PCR product can be sequenced, and that resulting sequence compared to known voucher sequences to complete or confirm a diagnosis. (Samples isolated from previously undescribed hosts are always sequenced as a means of confirmation.)

Note: There is a known potential problem of false positives being identified with the Nested PCR when *P. foliorum*, *P. hibernalis*, or *P. lateralis* DNA is present in a sample. After initial validation of the Nested PCR assay, it was discovered that under certain conditions (unusually high DNA titer of the target organism), or if the validated protocol is not followed exactly (*e.g.*, Bloomquist *et al.*, 2005), then the Nested PCR assay can also react irregularly to produce a Nested PCR product from *P. hibernalis*, *P. lateralis*, or from the recently discovered *P. foliorum* (species description in press). With all three of these non-target *Phytophthora* species, the Multiplex PCR reaction will generally react to produce a strongly amplifying *Phytophthora*-specific band, while the Nested PCR can produce a band of variable intensity. In these cases, samples must be resolved using an additional diagnostic technique, such as by obtaining sequence data for either the Multiplex or Nested (if there is sufficient quantity) target PCR products.

If the host is not listed on PPQ's Known Host or Associated Host list, PCR products must be sequenced.

If the sample is from a previously certified nursery, unusual environmental find, or other non-routine situation, PCR products must be sequenced. Note: To be considered positive for *P.ramorum* a sequence result must be ≥98% match.

10. Samples for Federal confirmation

A **Potentially Actionable Suspect Sample (PASS)** is a sample from a potential new location, a putative new host, a previously certified nursery, a geographically distinct/unique environmental find, or other non-routine situation. For more information, http://www.aphis.usda.gov/plant_health/plant_pest_info/pram/downloads/pdf_files/passpolicy4-06.pdf

A PASS sample must be forwarded to a federal lab for confirmation (see submission information below).
Samples are submitted by overnight delivery and must be accompanied by (1) a PPQ Form 391 (Sample Submission Form), (2) labeled gel photos and/or Ct values for each run (see example table below).

Prior to sending material please notify Dr. Palm by e-mail (Mary.Palm@aphis.usda.gov) with the tracking number and estimated delivery date using the shipping address below:

USDA-APHIS-PPQ-PHP-MDL
BARC-East, Bldg 580
Powder Mill Rd
Beltsville, MD 20705
Phone 301-504-5700

References:

Multiplex PCR: Winton, L.M. & E.M Hansen. 2001. Molecular diagnosis of *Phytophthora lateralis* in trees, water, and foliage baits using multiplex polymerase chain reaction. *Forest Pathology* 31:275-283.

Nested PCR: Hayden, J.H., D. Rizzo, J. Tse & M. Garbelotto. 2004. Detection and quantification of *Phytophthora ramorum* from California forests using a real-time polymerase chain reaction assay. *Phytopathology* 94:1075-1083.

Levy, L., and V. Mavrodieva, (May 2003). Evaluation of the PCR detection and DNA isolation methods for use in the *Phytophthora ramorum* National Pilot Survey (laboratory manual). http://ceris.purdue.edu/napis/pests/sod/natplan/may03-pcr.doc.

Cautionary Note: Bloomquist, C., T. Irving, N. Osterbauer & P. Reeser. 2005. *Phytophthora hibernalis*: A new pathogen on *Rhododendron* and evidence of cross amplification with two PCR

detection assays for *Phytophthora ramorum*. *Plant Health Progress* doi:10.1094/PHP-2005-0728-01-HN.

Document Revision History

Status (Original/Revision/Cancelled)	Document Revision Number	Effective Date	Description
Original	Original	4-3-2006	Conventional PCR assays for detection of *Phytophthora ramorum*
Revision	1	6-04-2007	Add requirement for positive nested reactions to be retested at both 1:10 and 1:100 dilutions. Added section 10 on PASS policy and federal lab (confirmation) information. Removed names of NPGBL-specific cycler programs.

Approved: Signature on File with Original Document
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